# Genome and metabolomic based approches to authenticate *Swertia chirata* from its adulterants and their comparative evaluation against hypoxia induced oxidative stress

**SYNOPSIS**

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**By**

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## Introduction

*Swertia chirayita* (Gentianaceae), a popular medicinal herb indigenous to the temperate Himalayas which is used in traditional medicine to treat numerous ailments such as liver disorders, malaria, and diabetes and are reported to have a wide spectrum of pharmacological properties. It's medicinal usage is well-documented in Indian pharmaceutical codex, the British, and the American pharmacopeias and in different traditional medicine such as the Ayurveda, Unani, Siddha, and other conventional medical systems. This ethnomedicinal herb is known mostly for its bitter taste caused by the presence of different bioactive compounds that are directly associated with human health welfare.

### Botanical Classification

The genus *Swertia* comprises 170 known species. The botanical classification of this genus reads as follows: family- Gentianaceae, tribe- Gentianeae, subtribe- Swertiinae, genus- *Swertia*. Most of the species found in India grow at high altitude in the temperate Himalayas from Kashmir to Bhutan, and also in the Khasia and Western Ghats hills (Chopra et al., 1956). Moreover, 97 *Swertia* species have been reported to be distributed in the main land China.

### Medicinal and pharmaceutical properties

*S. chirayita* is used as antipyretic, anthelmintic, antiperiodic, cathartic and in asthma and leucorrhoea in Ayurveda and as harsh, analeptic, stomachic, mitigate inflammation, relaxing to the pregnant uterus and never ending fevers (Kirtikar and Basu, 1918). It is a remedy for ulcers, Gastrointestinal diseases, skin diseases, cough, hiccup, liver and Kidney diseases, Neurological disorders, and urinogenital tract disorders. Also used as a purifier of Breast milk, and as a laxative and carminative (Garg, 1965; Sharma, 1986). *Swertia chirayita* is known for its medicinal and pharmaceutical importance. It is a filthy provenance of alkaloids and flavanoids, most of them having ample scale exercise. Their roots have considerable antipyretic and analgesic effects and a high rise therapeutic clue. It is having a large number of chemical constituents estimating more than twenty polyhydroxylated xanthones and some of these are swertinin, swerchirin, mangiferin, decussatin, and isobellidifolin; a dimeric xanthone and chiratanin have also been segregated (Bhattacharya et al., 1976). Important photochemicals like

Amarogentin and Swerchirin have been investigated for drug reinforcement (Brahamchari et al 2004).

**Adulteration and substitution**

*Swertia chirayita* has an organized vend both domestic in India as well as globally and it is increasing at an estimate of 10% every year. However, as the plant is still collected from wild, the existing population of *S. chirata* is diminishing and hence has been categorized as critically endangered according to the criteria of the new international union for conservation of nature and natural resources. Due to high demand in pharmacology and paucity in trade of *S.* chirayita the other *Swertia* species such as *S. angustifolia, S. paniculata, S. ciliata, S. cordata* including *Andrographis paniculata* are often misused or used as substitutes of *S. chirata* and thus affects the trade and economics of *S. chirata*. Global market of medicinal plants has a severe problem of erroneous substitutes and adulterants which are traded due to their lesser cost or misidentification of species with similar morphological features. Adulteration and substitution of herbal drugs is the major problem causing a threat to the herbal drug industry and to the research on commercial natural products. There are presently no global standard protocols or practices to identify and evaluate the different plant species or plant parts used in herbal products. It is only in recent years that the adverse consequences of species adulteration on the health and safety of consumers have come into the knowledge. Although, *S. chirayita* has been receiving increasing attention from a wide range of researchers as evident from the number of publications appearing in the literature. But for authentication of *S. chirata* few criteria and certain traditional methods have been developed which are mainly based on genetic phenotypes and analysis of chemical components. However, all the available methods used are affected due to intrinsic and extrinsic factors such as time of harvest, availability of experts and processing methods, etc. In recent years, the new diagnostic technique known as DNA barcoding has evolved which can be used as an efficient tool for the species identification. This approach includes sequencing of short standard DNA region from a small fragment of the genome, which can be amplified by universal primers.

**Oxidative Stress**

Oxidative stress plays a central role in the development and progression of a variety of complications such as hepatotoxicity, neurotoxicity, diabetes, Alzheimer's disorder, etc.

(Abdel-Salam et al., 2016; Reddy et al., 2009). To date, there is no drug available which can counter oxidative stress and associated complications. There is an urgent need for the screening of some safer and alternative therapeutic strategies which can effectively counter oxidative stress and therefore may attenuate associated complications. *S. chirata* has long been used in Ayurvedic and Unani medicine as an antihelminthic, febrifuge, stomach and liver tonic (Mehta et al., 2017; Kumar and Van, 2016).This plant is abundant with a large number of alkaloids and flavonoids along with other different phytoconstituents that are responsible for its all broad range of medicinal properties (Roy et al., 2015). *Swertia chirata* and *S. cordata* are well known medicinal plants in Ayurveda, which are known for their beneficial effects during hepatitis, liver disorders, inflammation, chronic fever, malaria, anemia, GIT disorders, hypertension, mental disorders, and diabetes (Kumar and Van, 2016, Roy et al., 2015). Till date, numerous scientific study have experimentally justified the traditional use of these plants for hepatic and other disorders (Khan et al., 2012), however, their use in the mental disorders remain unexplored so far, besides, the mechanisms through which the beneficial effects on the brain are achieved remain unknown. In this study, we aimed to provide scientific evidence to the traditional use of *S. chirata* and investigated whether or not plant extract is beneficial in the management of hypoxia-induced hepatotoxicity and investigated its effect on oxidative stress as a potential mechanism.

Thus, considering the above mentioned problems the study has been conducted with the following objectives:

1. Evaluate the feasibility of using candidate DNA barcodes to discriminate S*wertia chirata* from its adulterants.
2. Comparative metabolomics reveals the metabolic variation between *Swertia chirata* and its adulterants.
3. Antioxidant and hepatoprotective effect of S*wertia chirata* on hypoxia-induced oxidative stress in Wistar rats.
4. Hydroalcoholic extract of S*wertia chirata* and *Swertia cordata* attenuate hypoxia mediated memory dysfunction by improving neuronal survival in Wistar rats.

**Objective I**

**Evaluating the feasibility of using candidate dna barcodes in discriminating *Swertria chirata* from its adulterants**

**Materials and methods**

**Plant material**

Thirteen specimens belonging to six species of *Swertia* (Gentianaceae) were sampled for the present study from July to January 2011–2013. *Andrographis paniculata,,*and *Picrorhiza kurroa* used in this study were collected from Dhauhladar range of trans Himalayan region and greenhouse respectively. The species were identified and authenticated by Mr. Kisan Lal florists of India and Dr. Narain Singh Chauhan. Taxa taken for the study were listed in Table 1.1 with accession numbers of the DNA sequences.

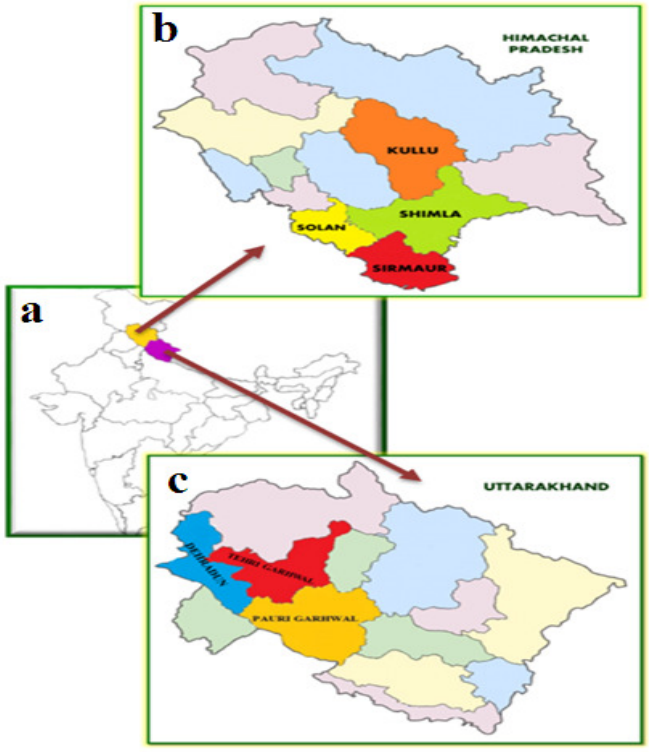


**Figure 1.1:** Natural distribution of *Swertia chirayita* (a). The shaded area represents the natural habitat of *Swertia chirayita* in the Himalayan Region. Shaded area represents collection of samples from the different regions from district Himachal (b) collection of samples from different places of Uttarakhand region (c).

**DNA extraction, Amplification, and Sequencing**

The genomic DNA was extracted from all the samples using Pure Link Plant Total DNA Purification Kit (Invitrogen) following the given protocol. The isolated DNA was used to obtain amplification of the candidate regions i.e rbcl, MatK, rpoC, rpoB, PsbK-I, atpH-F, psbA-trnH, ITS1-ITS2. The amplification was obtained for all the candidate regions using the primer sequences and reaction conditions given in table 1.1.

**Table 1.1:** Primer sequences and reaction conditions.

| Marker | Primer Sequence (5'-3') | Amplicon size | Ta Optimum |
|---|---|---|---|
| psbK-psbI | Forward: TTAGCATTTGTTTGGCAAG | 544 bp | 51°C |
| | Reverse: AAAGTTTGAGAGTAAGCAT | | |
| trnH-psbA | Forward: GTTATGCACGAACGTAATGCTC | 300 bp | 55°C |
| | Reverse: CGCGCGTGGTGGATTCACAATCC | | |
| matK | Forward: CGTACTGTACTTTTATGTTTACGAG | 862 bp | 55°C |
| | Reverse: ATCCGGTCCATCTAGAAATATTGGTTC | | |
| atpF-atpH | Forward: ACTCGCACACACTCCCTTTCC | 675 bp | 53°C |
| | Reverse: GCTTTTATGGAAGCTTTAACAAT | | |
| rpoB | Forward: ATGCAGCGTCAAGCAGTTCC | 406 bp | 55°C |
| | Reverse: TCGGATGTGAAAAGAAGTATA | | |
| rpoC1 | Forward: GGAAAAGAGGGAAGATTCCG | 509 bp | 56°C |
| | Reverse: CAATTAGCATATCTTGAGTTGG | | |
| rbcL | Forward: GTAAAATCAAGTCCACCACG | 580 bp | 56°C |
| | Reverse: ATGTCACCACAAACAGAGACTAAAGC | | |

The PCR conditions were same for all the candidate regions and the PCR products were examined using 2% agarose gel electrophoresis in 1xTAE buffer at 100 v for 45min.

**Sequence alignment and data analysis**

Sequences obtained were assembled and aligned with the help of CLUSTALX program and were adjusted manually in BioEdit (version 7.0.5). The sequence data obtained

for all the candidate regions of all the species were submitted in Gen bank Nucleotide database with sequence length and accession numbers are given in table 1.2.

**Table 1.2:** Accession numbers of the species used in the study

| Accession no. | *Species* | Accession no. | *Species* |
|---|---|---|---|
| KC594650 | *Aconitum heterophyllum* | KC594657 | *Picrorhiza kurroa* |
| KC594655 | *Andrographis paniculata* | KC594652 | *Andrographis paniculata* |
| KC594666 | *Swertia chirata* | KC594668 | *Swertia chordate* |
| KC594671 | *Swertia chordate* | KC594651 | *Aconitum heterophyllum* |
| KC594677 | *Swertia ciliate* | KC594656 | *Andrographis paniculata* |
| KC594660 | *Picrorhiza kurroa* | KC594667 | *Swertia chirata* |
| KC594653 | *Andrographis paniculata* | KC594672 | *Swertia chordate* |
| KC594664 | *Swertia chirata* | KC594661 | *Picrorhiza kurroa* |
| KC594669 | *Swertia chordate* | KC594649 | *Aconitum heterophyllum* |
| KC594675 | *Swertia ciliate* | KC594665 | *Swertia chirata* |
| KC594658 | *Picrorhiza kurroa* | KC594676 | *Swerita ciliate* |
| KC594653 | *Andrographis paniculata* | KC594649 | *Aconitum heterophyllum* |
| KC594664 | *Swertia chirata* | KC594670 | *Swertia chordate* |
| KC594648 | *Aconitum heterophyllum* | KC594659 | *Picrorhiza kurroa* |
| KC594663 | *Swertia chirata* | KC594670 | *Swertia chordate* |
| KC594674 | *Swerita ciliate* | | |

The data analysis was done for all the Gentianaceae sequences to evaluate the feasibility of candidate regions that can be used as barcode for the identification of species used in the study. The nucleotide diversity was examined of all the candidate DNA regions among all the species with the help of DNAsp software. The sequences were aligned using Clustal Omega and the genetic distances were calculated using MEGA 5.0 according to Kimura 2-parameter (K2P) (Tamura et al., 2011). The candidate regions were evaluated based upon the genetic divergence calculated using six parameters. The six parameters were used to calculate interspecific divergence and intraspecific variability. To characterize inter-specific divergence three parameters were used (1) average interspecific distance in each genus with at least two species, (2) mean pairwise distance i.e theta prime within each genus, (3) minimum interspecific distance within each genus. Further to calculate intra-specific variation additional

three parameters were determined: (1) average intra-specific distance within each species, (2) theta i.e mean pairwise distance within each species, (3) coalescent depth i.e maximum intra - specific distance within each species (Lahaye et al., 2008; Chen et al., 2010; Meier et al., 2008; Meyer & Paulay, 2005; Pang et al., 2010). The interspecific and intraspecific divergence was compared to evaluate the barcoding gap. In addition to this sequence data was analyzed and compared to test the potential of barcode regions to discriminate the species with the help of few most recommended methods (neighbor joining tree based on K2P distance and unweighted pair group method with arithmetic mean).

**Results**

In the present study successful amplification was obtained using universal primers in most of the candidate barcode regions among all species. The amplification success rate was maximum with psbk-psbI region followed by ITS1-ITS2, atpF-H, rbcl, matK, rpoC, rpoB. However, few regions were relatively difficult to amplify in case of few species. We were able to examine package, v5. For all the candidate regions genetic divergence within and between species was calculated using the Kimura 2-parameter model, which is accepted as the best model for analysis at significant DNA polymorphism among the sequences of various species using DNAsp software species level with low distances. All the three interspecific divergence parameters were compared to investigate the utility of all the candidate DNA regions to be used for the identification of *swertia* species and its adulterants. To discriminate species an ideal barcode region should possess high inter specific divergence in comparison to intra specific divergence. Measurement of inter- versus intra-specific genetic divergence at each locus Six metrics were employed to characterize inter- versus intra-specific variation. Psbk-I and atpf-H both exhibited significantly higher levels of inter-specific discriminatory ability than psbA-trnH, rpoB, rbcL, ITS and matK. The lowest divergence between specific individuals, as determined by all inter-specific calculations was exhibited by rbcL. The results of the intra-specific differences were similar, with atpf-H contributing the largest and rbcL the smallest variations (Fig. 1.2).

A favorable barcode should possess a high inter-specific divergence to distinguish different species. Barcoding studies normally use phylogenetic trees to assign species names, and the most commonly used tree is neighbor-joining, in which the assessment is based on phenetic distance and the evolutionary information of a species (Liu et al., 2012). The Neighbor Joining trees (Fig. 1.3) was used to evaluate the ability of these DNA barcodes to

identify species. All authenticate species clades were clearly monophyletic and distinct from other clades in the psbK-I trees. However, the Neighbor Joining trees for ITS2 and atpF-H did not correctly identify all of the relationships in *Swrtia* species and its adulterants. Based on the neighbor joining tree were constructed (Fig. 1.3), psbK-I barcode marker successfully separated each genus by representing monophyletic clades, in which each clade appeared distinctly distant from other clades.

**Table 1.3:** Single Nucleotide polymorphism obtained in PsbK-I using DNAsp

| PSBK-I (Forward) | 83 | 109 | 210 | 399 | 424 |
|---|---|---|---|---|---|
| *S. Ciliata* | a | g | c | c | g |
| *S. Paniculata* | a | g | c | c | g |
| *S. Angustifolia* | a | g | c | c | g |
| *S. Cordata* | c | g | g | c | g |
| *S. Chirata* | a | a | c | c | a |
| *A. Paniculata* | a | g | c | t | g |

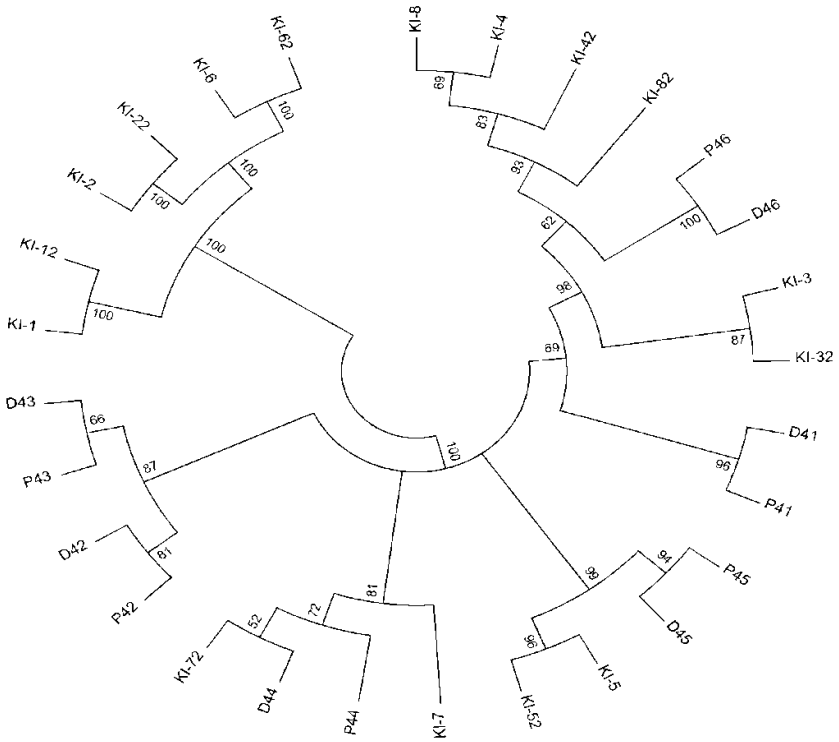


**Figure 1.3:** K2P distance NJ tree for psbK-I . A consensus NJ tree for *swertia chirata* and its adulterants assessed with 1000 bootstrap replicates was constructed by bootstrap analyses with the bootstrap values indicated at the branches (bootstrap values of less than 50 are not shown).

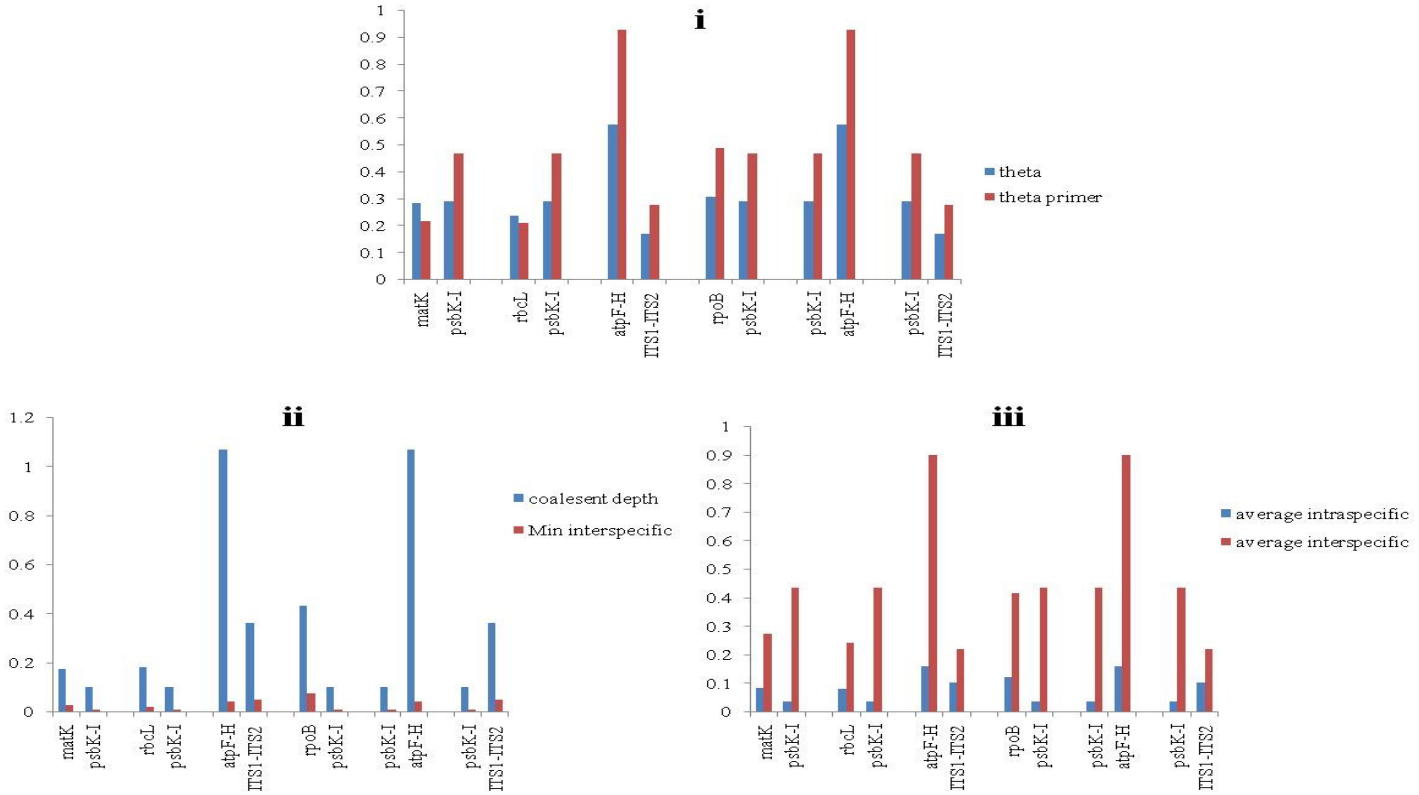


**Figure 1.4:** Analyses of the inter-specific divergence between congeneric species and intra-specific variation of the six loci. First, three parameters were used to characterize inter-specific divergence: (i) average inter-specific distance (K2P distance) between all species in each genus with at least two species; (ii) average theta prime (θ'), where theta prime is the mean pairwise distance within each genus with more than one species, thus eliminating biases associated with different numbers of species among genera; and (iii) smallest inter-specific distance, i.e., the minimum inter-specific distance within each genus with at least two species. Second, three additional parameters were used to determine intraspecific variation: (i) average intra-specific difference (K2P distance), that between all samples collected within each species with more than one individual; (ii) theta (θ), where theta is the mean pairwise distance within each species with at least two representatives; θ eliminates biases associated with unequal sampling among a species; and (iii) average coalescent depth, which is the maximum intra-specific distance within each species with at least two individuals

## Objective II

## Comparative metabolomics reveals the metabolic variation between swertia chirata and its adulterants

### Materials and Methods

### Plant Material

The samples were collected from the month of July-September. The species were identified and authenticated by Mr. Kisan Lal florists of India and Dr. Narain Singh Chauhan. The whole plant was shade dried and was finely powdered by using an electric blender and stored in a plastic bag container for further use at room temperature.

### Preparation of extracts

A portion of the dried sample was placed in a Soxhlet apparatus. Extraction was performed with 750 ml of methanol for 48 h at a temperature not exceeding the boiling point of the solvent. The extract was filtered through a 45 μm filter. The resulting solution was concentrated in vacuum to dryness to give methanol extract. The extract was stored in a refrigerator at 4°C for further use.

### GC-MS (Gas Chromatography-Mass Spectrometry) analysis

The phytochemical investigation of methanolic extract was performed on GC-MS equipment (Thermo Scientific Co.) Thermo GC-TRACE ultra ver.: 5.0, Thermo MS DSQ II. Experimental conditions of GC-MS system were as follows: TR 5-MS capillary standard non-polar column, dimension: 30Mts, ID: 0.25 mm, Film thickness: 0.25μm. Flow rate of mobile phase (carrier gas: He) was set at 1.0 ml/min. In the gas chromatography part, temperature program (oven temperature) was 40°C raised to 250°C at 5°C/min and injection volume was 1 μl. Samples dissolved in chloroform were run fully at a range of 50-650 m/z and the results were compared by using Wiley Spectral library search program.

### Statistical Analysis

The statistical analysis was performed using SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) was carried out to compare the content differences of species used in the study. A $P$ value $< 0.05$ was considered statistically

significant. To maximize the identification of differences in metabolic profiles between groups, Principal Component Analysis (PCA) was employed. Potential biomarkers were extracted from the values of the Variable Importance Plot (VIP). Heat maps and hierarchical cluster analyses were conducted using MeV version 4.9.0 software.

**Results**

The preliminary phytochemical study revealed that methanolic extract of *Swertia species* contains alkaloids, cardiac glycosides, flavonoids, phenols, phlobatannin, reducing sugars, saponins, steroids, tannins, terpenoids, volatile oils, carbohydrates, protein, and amino acids. Anthraquinones were absent in the *Swertia chirata* methanolic extract, as summarized in Table 2.1. The results pertaining to GC-MS analysis of the methanolic extract of all the sample species lead to the identification of a number of compounds. These compounds were identified through mass spectrometry attached with GC. The GC-MS spectrum confirmed the presence of various components with different retention times as illustrated in Figure 2.1. The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearance of peaks at different m/z ratios. These mass spectra are fingerprint of that compound which can be identified from the data library.

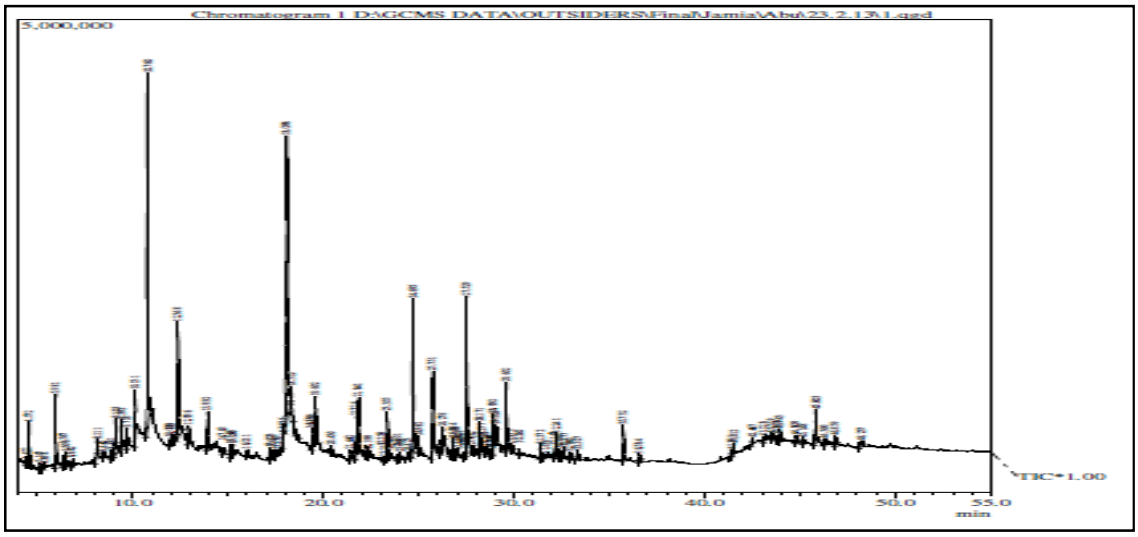


**Figure 2.1:** GC-MS Chromatogram showing different peaks at different retention time

Figure2.2b was a heat map showing the average normalized quantities of the metabolites present in all samples. As an unsupervised multivariate analysis method, the PCA

model provided an overview of metabolomic data of all the species. In the PCA model, sample dots with similar metabolomic compositions were clustered together while different metabolic components were dispersed. It was observed the three species which found that different components from each other as were dispersed on PCA plot.

A supervised OPLS-DA analysis technique was used to search biomarkers based on the VIP (Variable importance in projections) between the three species i.e *Swertia chirata, Angrographis paniculata and Swertia chordate*. The VIP values larger than one indicated the importance of the variables. Figure 2.5b showed the metabolites sorted from high to low according to the VIP values and their weighted sum of absolute regression coefficients (coef).

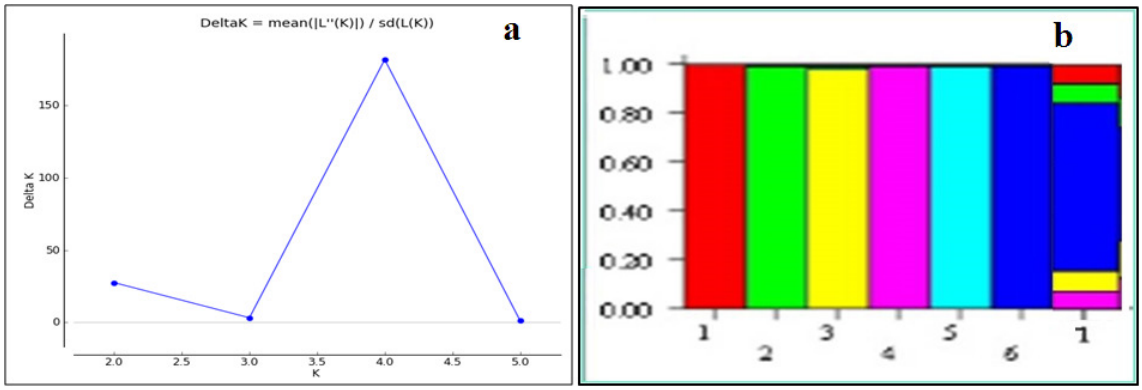


**Figure 2.2:** Graph plot between Delta K and K (a); Heat map of different species of *Swertia chirata* along with *Andographis paniculata* and Mixture (b).

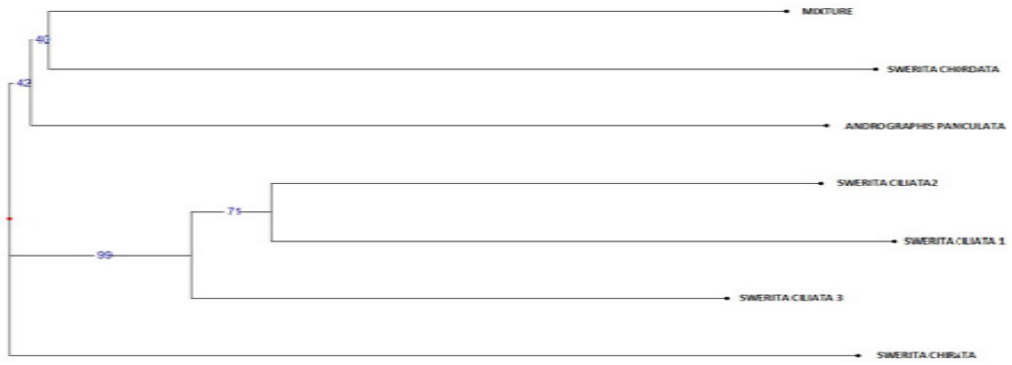


**Figure 2.2:** Dendrogram showing the evolutionary relationship between different species: *Swertia ciliata, Swertia chirata, Swertia chordata, Andrographis paniculata* and Mixture

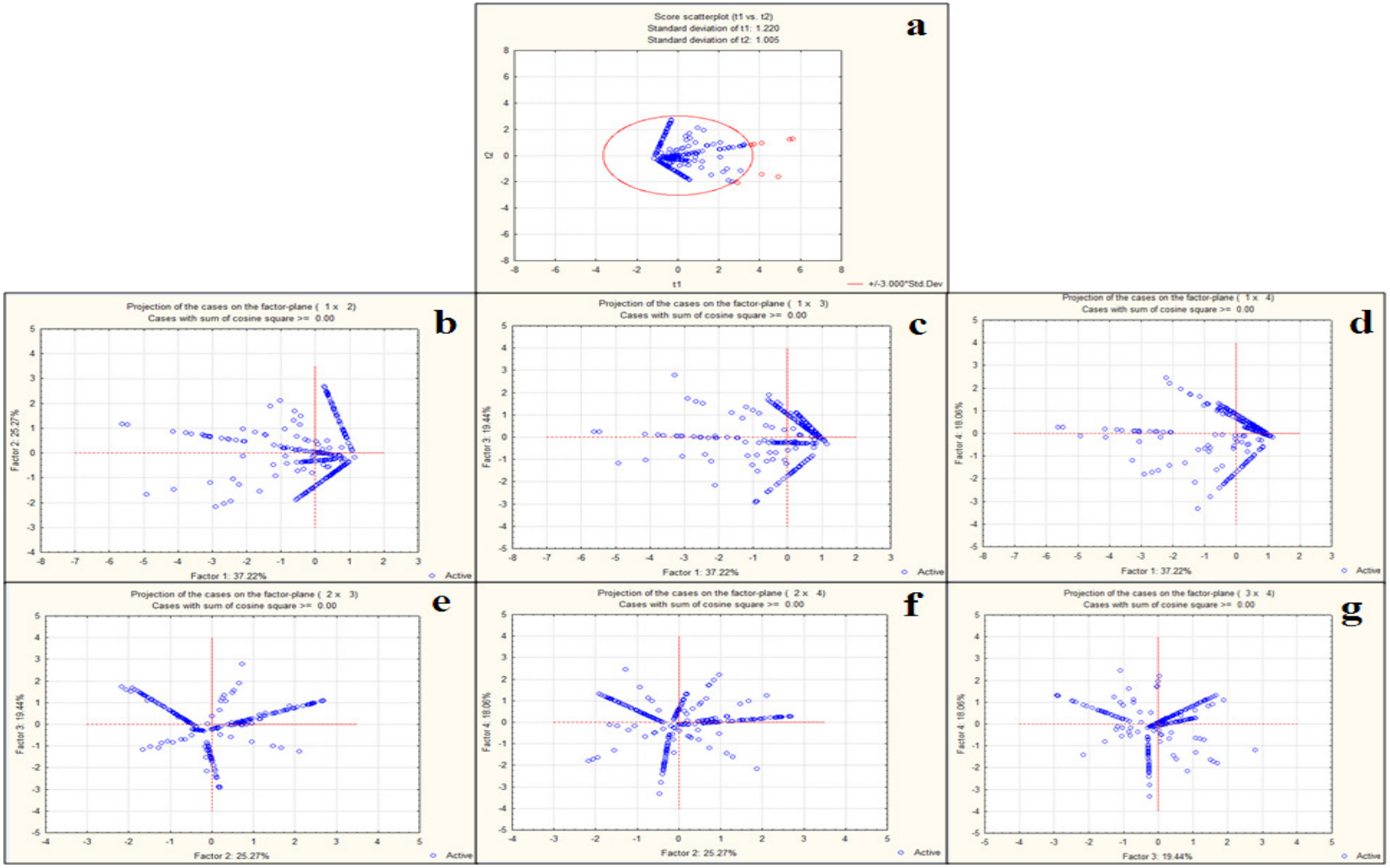
a
Score scatterplot (t1 vs. t2)
Standard deviation of t1: 1.220
Standard deviation of t2: 1.005
t1
t2
+/-3.000*Std.Dev
b
Projection of the cases on the factor-plane ( 1 x 2)
Cases with sum of cosine square >= 0.00
Factor 1: 37.22%
Factor 2: 25.27%
Active
c
Projection of the cases on the factor-plane ( 1 x 3)
Cases with sum of cosine square >= 0.00
Factor 1: 37.22%
Factor 3: 19.44%
Active
d
Projection of the cases on the factor-plane ( 1 x 4)
Cases with sum of cosine square >= 0.00
Factor 1: 37.22%
Factor 4: 18.06%
Active
e
Projection of the cases on the factor-plane ( 2 x 3)
Cases with sum of cosine square >= 0.00
Factor 2: 25.27%
Factor 3: 19.44%
Active
f
Projection of the cases on the factor-plane ( 2 x 4)
Cases with sum of cosine square >= 0.00
Factor 2: 25.27%
Factor 4: 18.06%
Active
g
Projection of the cases on the factor-plane ( 3 x 4)
Cases with sum of cosine square >= 0.00
Factor 3: 19.44%
Factor 4: 18.06%
Active

Figure 2.4: scatter plots of different species *S.Cordata,* 2- *S. Chirata,* 3- *A.Paniculata,* 4- *S. Ciliata*

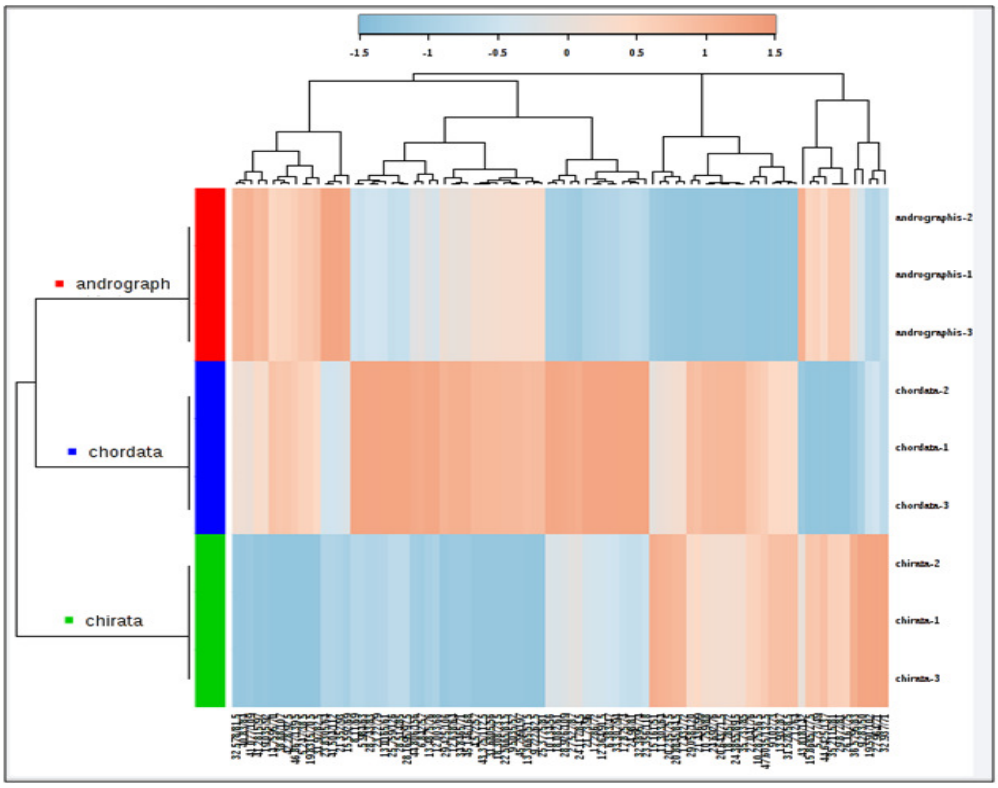


**Figure 2.5**: Heat map of metabolites in three groups. Where rows represent the metabolites and columns represents the samples in groups.

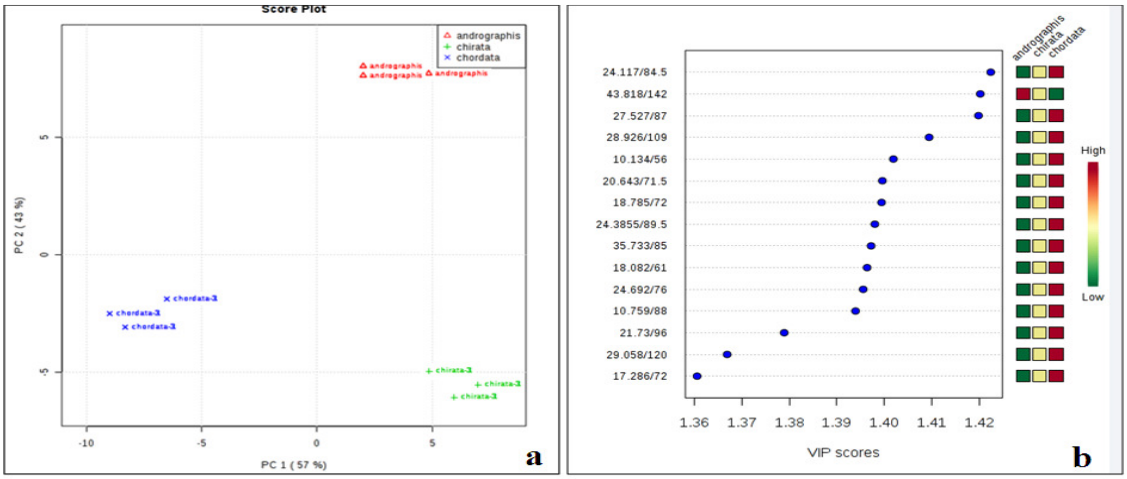


**Figure 2.6:** Principle component analysis (PCA) score plot (a) and variable importance in projections (VIP) scores (b) of 3 species of *Swertia.*

The VIP plot in OPLS-DA. The VIP plot is sorted from high to low. The colored boxes on the right indicate the concentrations of the corresponding metabolite in each group under study. The dotted line indicates critical level of VIP values.

**Objective III**

**Antioxidant and hepatoprotective effect of S*ertia chirata* on hypoxia-induced oxidative stress in Wistar rats**

## Materials and methods

### Material

All the chemicals and reagents used in this study were of analytical grade procured from Sigma, HiMedia, and LobaChemie., unless otherwise specified. Estimation of serum biochemical parameters was performed on Hitachi Modular P800 autoanalyzer. Kits for biochemical assays were obtained from Roche Diagnostics (Roche Diagnostics K.K., Tokyo, Japan). 5,50-dithiobisnitrobenzoic acid (DTNB) and 2-thiobarbituric acid (TBA) were obtained from the Sigma Chemical Co. (St. Louis, MO, USA).

### Sample collection and extraction

Plant species were collected from high altitude regions of Himachal Pradesh and Uttarakhand during the month of July–September. The plant was identified and authenticated by Prof. (Dr.) Narayan S. Chauhan, Dr. YS Parmar University of Horticulture and Forestry, Sola, Himachal Pradesh, India. The whole plant was used for the preparation of the extract. The samples were washed, shade dried at room temperature and coarsely powdered. Extraction was performed in a Soxhlet apparatus at 25°C for 72 h in 80% methanol. The extract was filtered while hot, concentrated under reduced pressure by rotary evaporator and lyophilized. Extract yield was calculated and the extract was stored at -20°C in a refrigerator until used for further analysis.

### Animals

All experimental protocols were performed on male Wistar rats after approval from the Institutional Animal Ethics Committee (DIHAR/IAEC/36/2015). All the experiments were designed and performed strictly in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India. Wistar rats (150-200g; 8-10 month age) were housed in the DIHAR animal house inside polypropylene cages (2 animals per cage) at the temperature of 23±2ºC, 12h day/light cycle and 65±5% relative humidity. Animals had free access to food and water. All necessary precautions were taken to minimize the sufferings to the animals.

**Animal Groupings**

Animals were divided into 6 groups having 5 animals in each group as follows.

Group 1- Control (CTRL) (received 0.3% carboxymethyl cellulose as a vehicle orally)

Group 2- CTRL + 50mg/kg*S. chirata* extract suspended in a vehicle (CTRL 50)

Group 3- CTRL + 100 mg/kg *S. chirata*extract suspended in a vehicle (CTRL 100)

Group 4- Hypoxia (HYP) (rats exposed to 10% oxygen for 3 days and received vehicle orally)

Group 5-HYP+50mg/kg*S. chirata* extract suspended in a vehicle (HYP 50)

Group 6-HYP+ 100 mg/kg *S. chirata*extract suspended in a vehicle (HYP 100)

All the treatments were given orally once daily between 6-7 pm for 7 days.

**Induction of hypoxia**

Hypoxic conditions were provided to the animals by placing them inside a custom-made hypoxia chamber linked with oxygen ($O_2$) and nitrogen ($N_2$) cylinders. The concentration of $O_2$ was regulated by infusing $N_2$ into the chamber. The hypoxic treatment was given to the rats in the hypoxia chamber at 10% $O_2$ for 3 days.

**Animal sacrifice and sample collection**

Animals were sacrificed by cervical dislocation and tissues were collected for biochemical and histopathological examinations. For biochemical evaluation, tissue samples were weighed, homogenized in HEPES buffers and stored at 4ºC until used. Blood was collected and serum was isolated for the evaluation of liver function tests (enzymes). All the samples were used within 6 h for the biochemical evaluations. For histopathological examinations, tissues were fixed by double circulation by infusing phosphate buffer saline (PBS; pH 7.4) followed by a fixing solution of 2% glutaraldehyde and 2 % formaldehyde. Tissues were collected and stored in fixing solution at room temperature.

**Biochemical evaluation**

**Isolation of Mitochondria**

Mitochondria were isolated using amethod described by Brown et. al. (2004), with slight modifications. Briefly, dissected tissue was weighed and homogenized byteflonhand homogenizer in homogenizing buffer (five volumes). Homogenizing buffer consisted of 1 mMethylene glycol tetraacetic acid (EGTA), 215 mMmannitol, 75 mM sucrose, 0.1 % bovine serum albumin (BSA), 20 mMhydroxyethylpiperazineethane-sulfonic acid (HEPES) and pH adjusted to 7.2 with potassium hydroxide. Homogenate was centrifuged at 10,000 g at 4ºC for

5 min. The resulting pellet was suspended in 500µl homogenizing buffer. In order to pellet out the mitochondria, centrifugation was carried out again at 13,000g for 10 min. The pellet obtained after centrifugation was washed in the EGTA containing buffer and centrifuged at 10,000g for 10 min, and suspended in the same buffer at a concentration of 10 mg/ml.

**Mitochondrial ROS Generation**

Mitochondrial ROS generation was evaluated in accordance with the method described by Wasilewski and Wojtczak (2005), with some necessary modifications. Briefly, approximately 10 µl of mitochondria (15 µg protein equivalent) were incubated with the respiration buffer containing 5 mM pyruvate, 2.5 mM malate, and 10M of dichlorodihydrofluoresceindiacetate ($H_2$DCFDA) for 20 min at 37ºC. After incubation, fluorescence as a result of ROS generationwas quantified using a CaryEclipsefluorimeter (Varian, Palo Alto, USA) (excitation 485nm, emission 582 nm) and related to total protein content. Moreover, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) was added to inhibit the interference from the membrane potential-dependent ROS production.

**Lipid Peroxidation**

Lipid peroxidation was assayed by the method of Mehta et. al. (2017), with some necessary modifications. Briefly, 500 µl of mitochondrial extract equivalent of 15 µg protein was diluted with equal volume of Tris-HCl buffer (0.1 M, pH 7.4). The reaction mixture was incubated for 2 h at 37$^{o}$C with constant shaking inside an incubator. 1000 µl 10% w/v of trichloroacetic acid was added to the reaction mixture, mixed thoroughly and subjected to centrifugation at 8000 rpm for 10 min. 1500µl 0.67 % w/v thiobarbituric acidwas added to each tube and tubes were heated to 100$^{o}$C for 10 min in a boiling water bath. Reaction tubes were cooled under running tap water, centrifuged at 10000 rpm for 15 min and the absorbance of the supernatant was recorded at 532 nm using UV spectrophotometer. The results were expressed as nmol TBARS/mg protein.

**Catalase activity**

Catalase activity was assayed spectrophotometrically at 240 nm and the results were expressed as moles of hydrogen peroxide ($H_2O_2$) consumed as per the method described by Mehta et. al. (2017). The reaction mixture consisted of a 500µl brain homogenate equivalent of 15 µg protein. The reaction was initiated by the addition of 32 µl of 30% $H_2O_2$. Absorbance was recorded every minute for 4 minutes using UV spectrophotometer and the

level of catalase in the homogenate was determined in terms of the amount of $H_2O_2$ consumed during the 4-minute reaction interval.

**Glutathione peroxidase assay**

Glutathione peroxidase activity was determined by the method previously described by Necheles et. al.(1969), with some modifications as per the requirements. The reaction mixture consisted of 400µl of 0.35 M phosphate buffer (pH 7.4), 100µl of 10 mM sodium azide, 200µl of 8 mM freshly prepared glutathione (GSH), 500µl mitochondrial sample equivalent to 15 µg protein, 100µl 2.5mM $H_2O_2$, and 1100µl double distilled water. The reaction mixture was incubated for 5 min at 37$^{o}$C. 500µl of 10% trichloroacetic acid was added to the reaction mixture followed by centrifugation at 3500 rpm for 15 min at room temperature. 500µl supernatant was diluted with 3000µl of 0.3 M disodium hydrogen phosphate. 1000µl 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB)(freshly prepared in phosphate buffer pH 7.4) was added to the reaction mixture. The reaction mixture was allowed to stand for 5 min at room temperature and then its absorbance was recorded at 412 nm using a UV spectrophotometer. The results were expressed as mg of GSH consumed/min/mg protein.

**Liver Function Test**

Serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and serum aspartate transaminase (ALP) are the extensively studied biomarkers of the hepatic damage in both, clinical as well as pre-clinical settings. Liver function testswere performed to evaluate the effect of *S. chirata* extract in hypoxia-induced liver damage in term of SGOT,SGPT and ALP levels. Levels of these enzymes were measured by using commercially available kits as per the manufacturer's instructions.

**Histopathological Examination**

Histopathological examination of the liver tissue was performed to evaluate the hypoxia-mediated liver damage and to evaluate the hepatoprotective potential of hydroalcoholic leaf extract of *S. chirata* as per the method described by Aboonabi et. al. (2014). Briefly, animals were anesthetized and tissue was fixed by double circulation technique by infusing phosphate buffer saline (pH 7.4) followed by a fixing solution of 2% glutaraldehyde and 2 % formaldehyde. Tissues were collected and stored in fixing solution at room temperature. Tissues were embedded into the paraffin wax and 5 µm thick section were

prepared by using microtome. Sections were stored at room temperature and histopathological alterations were detected by staining sections with hematoxylin and counterstaining with eosin (hematoxylin-eosin staining). Effect of hypoxia and extract treatment on cellular morphology of liver was assessed by analyzing images captured at 100X magnification.

**Statistical analysis**

All the statistical analysis were carried out byGraphPad Prism 6. All values are expressed as mean ± SEM. Statistical significance was determined by one way ANOVA followed by Dunnet post hoc test at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs HYP.

**Results**

**Extractive yield and phytochemical evaluation**

The extractive yield of the hydroalcoholic extraction of *S. Chirata* was observed to be 22.37% of the crude plant sample. Phytochemical screening was performed and it revealed the presence of flavonoids, alkaloids, glycosides, steroids, terpenoids, tannins and saponins. Volatile oil, fixed oil, proteins and acidic compounds were not present in the plant extract. Moreover, we observed the high amount of phenolic and flavonoids present in the plant extract which might be responsible for its antioxidant potential.

**Biochemical investigation**

**Mitochondrial ROS Generation**

Mitochondrial ROS generation and the effect of hydroalcoholic extract of *S. chirata* on it was determined and the results are depicted in Fig. 3.1 (A). Subjecting animals to hypoxic stress resulted in high levels of oxidative stress as indicated by the significantly ($p < 0.001$) high levels of ROS generated in the HYPgroup when compared to CTRL. ROS levels differed nonsignificantly in control animals treated with plant extract at 50 mg/kg and 100 mg/kg dose, when compared to CTRL, suggesting that plant extract is not associated with enhancing ROS production directly. Further, treating hypoxic rats with 50 mg/kg and 100 mg/kg dose of *S. chirata* extract significantly ($p < 0.01$) lowered the generation of mitochondrial ROS in a dose-dependentmanner when compared to HYP animals. These results suggest that *S. chirata* extract is having good antioxidant potential which may aid it the beneficial effects associated with extract treatment in the animals.

**Lipid Peroxidation**

Malondialdehyde (MDA) levels are used to determine the levels of lipid peroxidation in the biological samples, especially in the animal experimentation. We used this method to determine the effect of *S. chirata* extract treatment on the lipid peroxidation in hypoxic rats and the results are depicted in Fig. 3.1 (B). We observe a significantly ($p < 0.001$) high levels of MDA or lipid peroxidation in HYP animals when compared to CTRL, suggesting the development of oxidative stress in HYP animals. Treating HYP animals with plant extract resulted in a dose-dependent lowering of the lipid peroxidation levels at 50 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.01$) dose. We did not observe any significant lipid peroxidation in control animal treated with plant extract and the MDA levels in these groups were similar to CTRL, suggesting that plant extract is not associated with lipid peroxidation directly.

**Catalase activity**

Catalase is a strong antioxidant enzyme present in our body which is responsible for the detoxification of the ROS generated from the electron transport chain in the mitochondria. We evaluated the effect of *S. chirata* extract treatment on the catalase activity during hypoxia in Wistar rats and the results are depicted in Fig. 3.1 (C). Our results suggest that hypoxia leads to the saturation of catalase enzyme and its levels were observed to be significantly ($p < 0.001$) lower in HYP animals when compared to CTRL. Treat hypoxic animals with the plant extract resulted in a dose-dependent improvement in catalase levels and catalase activity was observed to be significantly higher in HYP animals treated with 50 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.01$) plant extract when compared to CTRL. Catalase levels were observed to be normal in control animals treated with extract when compared to CTRL. These results suggest that *S. chirata* is having an antioxidant potential which can be partially attributed to its potential to either upregulate catalase activity or prevent its depletion during oxidative stress.

**Glutathione peroxidase assay**

Determination of the glutathione levels is an established method to estimate the level of oxidative stress in the body. We evaluated the effect of *S. chirata* extract treatment on the glutathione during hypoxia in Wistar rats and the results are depicted in Fig. 3.1 (D). Our results suggest that hypoxia leads to marked oxidative stress as depicted by the significantly ($p < 0.01$) reduced glutathione levels in HYP animals when compared to control. Treating hypoxic animals with the plant extract resulted in a dose-dependent elevation in glutathione

levels at 50 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.05$), when compared to HYP. Normal animals treated with extract showed glutathione levels comparable to control.

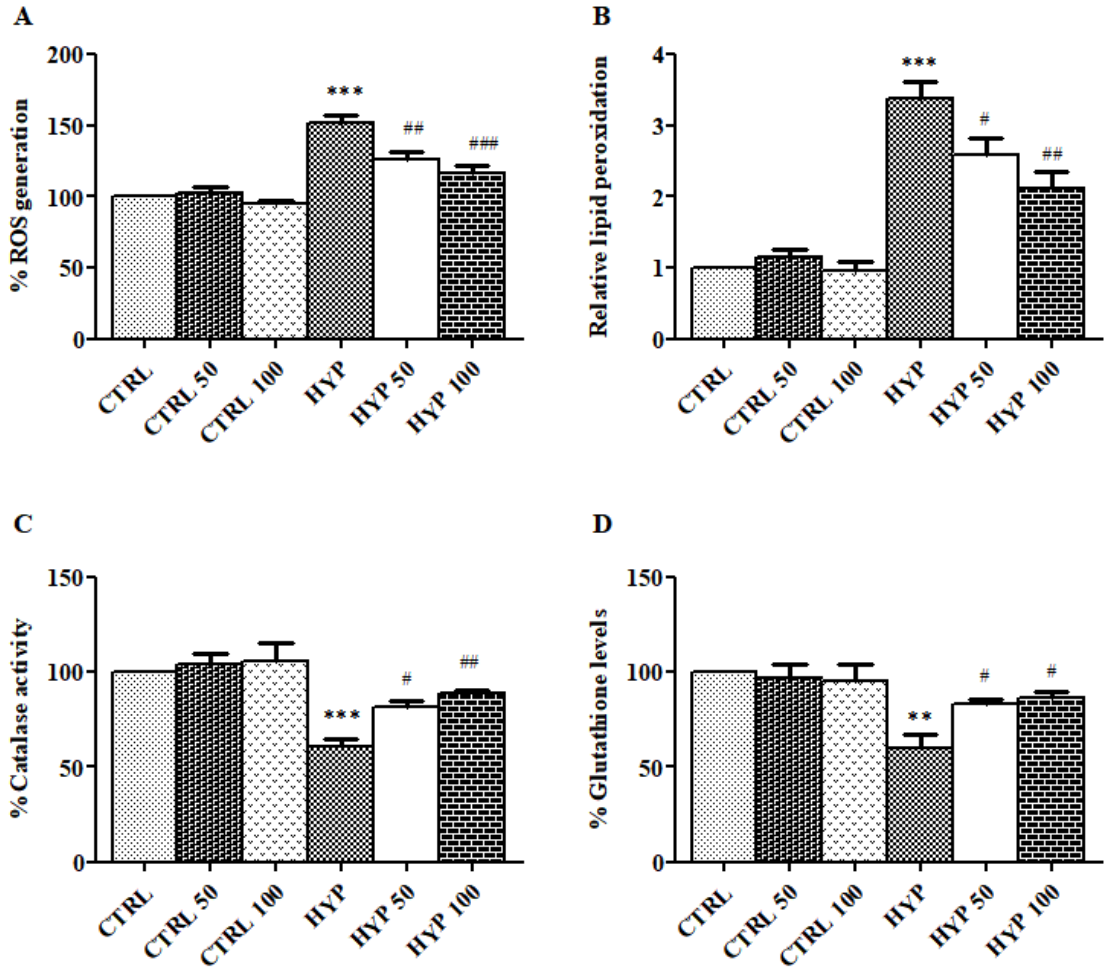


**Fig. 3.1:** Effect of *S. chirata* treatment on hypoxia induced oxidative stress (A- % ROS generation; B- lipid peroxidation; C- % catalase activity; D- % glutathione levels). Values are represented mean ± SEM. CTRL- control; CTRL 50- control + 50 mg/kg extract; CTRL 100- control + 100 mg/kg extract; HYP- hypoxia; HYP 50- hypoxia + 50 mg/kg extract; HYP 100- hypoxia + 100 mg/kg extract. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus CTRL group. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus HYP group.

**Liver Function Tests**

Hypoxia-induced hepatic damage and the hepatoprotective effect of *S. chirata* treatment was determined by evaluating liver function tests in which we determined the biochemical levels of SGOT, SGPT,and ALP (Fig. 3.2). Hypoxia resulted in a significant ($p <$

0.001) increase in the serum levels of SGOT, SGPT,and ALP, indicating the marked damage of hepatic cells. Extract treatment exerted a dose-dependent effect on hepatic biochemical enzymes. Levels of SGOT, SGPT and ALP were reduced significantly by the extract treatment at 50 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.01$) extract treatment. These results suggest that extract treatment efficiently protected hepatic cells from hypoxia-mediated damage. Moreover, extract treatment had no hepatotoxic effect of its own as the levels of SGOT, SGPT and ALP were observed to be non-significantly different from control animals.

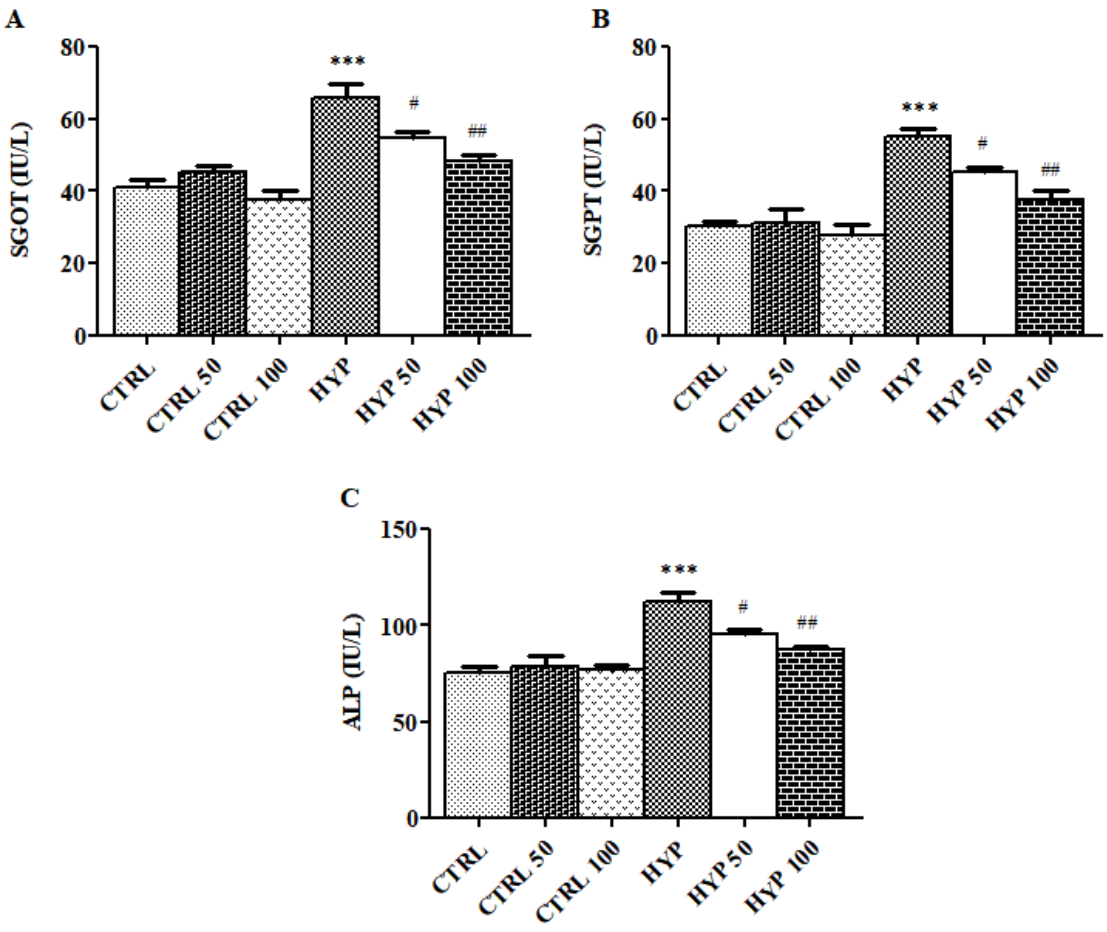


**Fig. 3.2:** Effect of *S. chirata* treatment on hypoxia induced hepatic damage (Liver Function Test). A- SGOT (IU/L); B- SGPT (IU/L); C- ALP (IU/L). Values are represented mean ± SEM. CTRL- control; CTRL 50- control + 50 mg/kg extract; CTRL 100- control + 100 mg/kg extract; HYP- hypoxia; HYP 50- hypoxia + 50 mg/kg extract; HYP 100- hypoxia + 100 mg/kg extract. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus CTRL group. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus HYP group.

**Histopathological Examination**

We further confirmed the hypoxia-mediated liver damage and the effect of extract treatment on it through histopathological examination of the Liver using hematoxylin-eosin staining. We observed a normal architecture of hepatic cells in control animals, cells appeared to be healthy and had a normal physiological appearance with no signs of damage or cytotoxicity. We further observed a marked liver damage in animals subjected to hypoxia (HYP). Cellular morphology was distorted and signs of significant cell death, cellular damage and necrosis were observed in these animals. Treating hypoxic animals with plant extract resulted in significant hepatoprotective effect, which was comparable at both the treatment dose. Hepatic cells appeared healthy with significantly lesser signs of cellular damage, cell death, andnecrosis when compared to HYP. Extract treatment efficiently protected hypoxia-mediated cellular damage in the liver. Moreover, *S. chirata* was not having any hepatotoxic effect of its own at the given doses as indicated by the normal cellular morphology and histopathological appearance of control animals treated with the plant extract which was comparable to control.

**Objective IV**

**Hydroalcoholic extract of *swertia chirata*and *swertia cordata* attenuate hypoxia mediated memory dysfunction by improving neuronal survival in wistar rats**

**Materials and methods**

**Chemicals and reagents**

Analytical grade chemicals and reagents used in this study. All the chemicals and reagents were procured from Sigma, HiMedia, and LobaChemie., unless otherwise specified on the first mention. Kits for the measurement of oxidative stress parameters were procured from Roche Diagnostics (Roche Diagnostics K.K., Tokyo, Japan). UV-Spectrophotometric recordings were taken on nano-drop spectrophotometer (ND-2000C, Thermo Scientific).

**Plant collection and extract preparation**

*S. chirata* and *S. cordata*whole plants were collected between July and September from the high altitude regions of Himachal Pradesh and Uttarakhand. Both plants was identified by local herbal medical practitioners and authenticated by Prof. N. S. Chauhan, Dr. Y. S. Parmar University of Horticulture and Forestry, Solan, (H. P.) India. Plants were washed to remove dirt, shade dried at room temperature till constant weight was achieved and coarsely powdered. Plants were subjected to hydroalcoholic extraction (80:20; methanol : water) inside Soxhlet extractor at 25°C for 72 h. Extracts of both plants was filtered while hot. It was then concentrated by using rotary evaporator and lyophilized. Extract yield was calculated and the extract was stored at -20°C in a refrigerator until used for further analysis.

**Animals**

Entire study was performed on male Wistar rats. Experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) (DIHAR/IAEC/36/2015). Experiments were performed strictly as per the guidelines of CPCSEA, Govt. of India. Wistar rats (150-200g; 8-10 month age) were housed in the DIHAR animal house inside polypropylene cages (2 animals per cage) under standard conditions of temperature (23±2ºC), 12h day/light cycle and relative humidity (65±5%). Animals had free access to food and water.

**Animal Groupings**

Animals were divided into following groups.

Group 1- Control (CTRL) (received 0.3% carboxymethyl cellulose as a vehicle orally)
Group 2- CTRL + 100 mg/kg *S. chirata* extract suspended in a vehicle (SCA 100)
Group 3- CTRL + 100 mg/kg *S. cordata*extract suspended in a vehicle (SCO 100)
Group 4- Hypoxia (HYP) (rats exposed to 10% oxygen for 3 days and received vehicle orally)
Group 5- HYP + 100 mg/kg *S. chirata* extract suspended in a vehicle (HYP-SCA 100)
Group 6- HYP + 100 mg/kg *S. cordata* extract suspended in a vehicle (HYP-SCO 100)

Each group had 5 animals and all the treatments were given orally once daily between 6-7 pm for 7 days as per the groupings. Behavioral studies were performed 24 h after the last dose to avoid interference from the possible acute drug effect.

**Hypoxia induction**

Animals were subjected to hypoxia inside a custom made hypoxia chamber. This chamber was linked to oxygen ($O_2$) and nitrogen ($N_2$) cylinders. The concentration of $O_2$ inside the chamber or the concentration of $O_2$ to which animals were exposed was regulated by $N_2$. In this study, hypoxia was inflicted by exposing rats to 10% $O_2$ for 3 days.

**Rota-rod test**

Effect of hypoxia and extract treatments on muscle coordination was evaluated through Rota rod test according to the method described by Mehta et al. (2017). Rota rod test is one of the most extensively used procedure to evaluate muscle coordination and strength in rodents. Animals were brought to the experimental room 30 min prior to the experimentation to get them acclimatize to the laboratory conditions. Each animal was placed on the rotating bar of the Rota rod (30 rpm) and time take to fall from the bar was recorded. Each animal was give three trials at an interval of 5 min with maximum cut-off time was 180 sec. The best time recorded was taken as the final reading to evaluate muscle coordination. Entire apparatus was wiped with 70% ethanol between every experimental session to avoid any sort of olfactory cues.

**Memory function tests**

**Passive avoidance test**

Effect of extract treatments on the hypoxia mediated memory dysfunction was evaluated through passive avoidance paradigm as per the method described previously (Mehta

et al., 2017a), with few modifications as per the requirements of our study. The apparatus consisted of two adjacent wooden chambers. One of the chamber was painted white and was brightly illuminated by 100 W bulb placed 3 feet above the chamber. Other chamber was painted black and was covered to provide dark environment to the animals. Both chambers were interconnected through a small opening (5 cm × 5 cm), which provided free access to the animals to enter any chamber. Animals were brought to experimental room, illuminated by low intensity light, 30 min prior to the start of learning trial. Each animal was placed in the light chamber and time taken to enter the dark chamber was recorded, with maximum cut-off or 180 sec. As soon as the animal entered the dark chamber, an inescapable foot chock was delivered through grid floor. Animals were immediately removed and returned to their home cages. Memory retention was evaluated 24 h after the learning trials. Animals were again placed in the light chamber and time taken to enter the dark chamber was recorded, with maximum cut-off or 180 sec. However, this time no foot shock was delivered. Entire apparatus was wiped with 70% ethanol between every experimental session to avoid any sort of olfactory cues.

**Active Avoidance Test**

Effect of hypoxia and extract treatments on memory acquisition and retention was evaluated through active avoidance paradigm as per the method described by Moscarello et al. (2013), with few modifications as per the requirements of our study. The apparatus consisted of two adjacent wooden chambers painted white. Both chambers were having grid floor capable of delivering foot shock and were interconnected through a small opening (5 cm × 5 cm), which provided free access to the animals to enter any chamber. In one of the randomly selected chamber, animals were given scrambled electric foot while the other served as a non-shock chamber. Animals were brought to experimental room, illuminated by low intensity light, 30 min prior to the start of learning experiment. Each animal was placed in the random chamber and were given conditioned stimulus through a buzzer for 2 s followed by a buzzer and foot shock of 0.1 mA, 40 V for 4 s. To avoid the foot shock, if rat moved to a shock-free chamber the avoidance was considered positive. If rat failed to move, it was gently guided to the shock-free chamber for earning. This process was repeated thrice and then animals were returned to their home cages. 24 h learning trials, animals were again brought to the experimentation room for memory retention trials. Each animal was placed into the random chamber of the apparatus and was given conditioned stimulus and the time taken to reach safe

chamber was recorded as the avoidance behavior, with the maximum cut-off of 180 sec. No foot shock was given during the retention trials. Entire apparatus was wiped with 70% ethanol between every experimental session to avoid any sort of olfactory cues.

**Animal sacrifice and sample collection**

For biochemical evaluation, animals were sacrificed by cervical dislocation, brain was dissected out, hippocampus was isolated, weighed, homogenized in HEPES buffers and stored at 4ºC until used. All the samples were used within 6 h for the biochemical evaluations. For histopathological examinations, animals were anesthetized with 90 mg/kg ketamine + 5 mg/kg Xylazine followed by fixation with 2% formaldehyde + 2% glutaraldehyde solution using double circulation technique. Tissues were collected and stored in fixing solution at room temperature until used further (Mehta et al., 2017a; Mehta et al., 2017b).

**Mitochondrial reactive oxygen species (ROS) Generation**

Effect of hypoxia and extract treatment on mitochondrial ROS generation was evaluated. As per the method described by Wasilewski and Wojtczak (2005), with required modifications. Briefly, amount of protein in the hippocampal homogenate was quantified by using Bradford method. 15 µg protein were incubated with the reaction buffer (5 mM pyruvate, 2.5 mM malate, and 10 M of dichlorodihydrofluoresceindiacetate) for 25 min at 37ºC. Level of fluorescence generated as a result of interaction of dichlorodihydrofluoresceindiacetate with ROS was quantified using a CaryEclipsefluorimeter (Varian, Palo Alto, USA) at excitation wavelength of 485 nm and emission wavelength of 582 nm. Results were normalized to protein content.

**Levels of Lipid Peroxidation**

Effect of extract treatment on hypoxia mediated lipid peroxidation was assayed according to the method of Mehta et. al. (2017a), with slight modifications. Briefly, hippocampal homogenate equivalent to 15 µg protein was diluted with equal volume of 0.1 M Tris-HCl buffer having pH 7.4. The resulting mixture was incubated for 2 h at 37$^{o}$C with constant mixing inside an incubator. One ml trichloroacetic acid (10% w/v) was added to the reaction mixture, mixed and centrifuged at 10000 rpm for 7 min. Further, 1.5 ml thiobarbituric acid (0.67 % w/v) was added to each tubes, followed by incubation over a water bath maintained at 950$^{o}$C for 10 min. Reaction tubes were cooled to room temperature,

centrifuged at 10000 rpm for 15 min, supernatant was collected and the absorbance was recorded at 532 nm using UV spectrophotometer. The results were expressed as nmol TBARS/mg protein.

**Catalase activity**

Effect of hypoxia and extract treatments on the hippocampal catalase activity was assayed as per the method described by Mehta et al. (2017a). Catalase activity was recorded spectrophotometrically at 240 nm and the results were expressed as moles of hydrogen peroxide ($H_2O_2$) consumed per minute. Hippocampal homogenate equivalent of 15 µg protein was diluted to 0.5 ml and 32 µl of 30% $H_2O_2$ was added to it. Absorbance was immediately recorded and readings were taken every minute for 4 minutes. The level of catalase in the hippocampal homogenate was determined in terms of the amount of $H_2O_2$ consumed during the 4-minute reaction interval.

**Glutathione levels**

Effect of hypoxia and extract treatments on the hippocampal glutathione peroxidase activity was determined by using the method of Necheles et al. (1969), with few modifications. Reaction mixture was prepared by mixing 0.4 ml phosphate buffer (0.35 M; pH 7.4), 0.1 ml sodium azide (10 mM), 0.2 µl freshly prepared glutathione (GSH) (8 mM), 0.5 ml hippocampal homogenate equivalent to 15 µg protein, 0.1 ml $H_2O_2$ (2.5 mM), and 1.1 ml double distilled water in respective tubes. These tubes were then incubated for 5 min at 37$^{o}$C followed by addition of 0.5 ml trichloroacetic acid (10%). Reaction mixture was centrifuged at 3500 rpm for 15 min at room temperature, supernatant was isolated and 0.5 ml supernatant was diluted with 3 ml of disodium hydrogen phosphate (0.3 M). 1 ml freshly prepared DTNB (in phosphate buffer pH 7.4) was added to the reaction mixture and the reaction mixture was allowed to stand for 5 min at room temperature. Absorbance was recorded at 412 nm using a UV spectrophotometer and the results were expressed as mg of GSH consumed/min/mg protein.

**Histopathological Examination**

Effect of extract treatments on the hypoxia mediated neuronal damage was evaluated through histopathological examination, as per the method described previously (Mehta et al., 2017a; Mehta et al., 2017b). Briefly, brain of the animals were processed as described above. Fixed brain samples were embedded into the paraffin wax and thick section of 5 µm were

prepared by microtome. Sections were fixed to glass slides, which were then stored at room temperature until used. Neuronal integrity and damage was detected by hematoxylin-eosin staining method. Images of the sections were captured at 100X magnification and the effect of hypoxia and extract treatments on neuronal morphology was determined.

**Statistical analysis**

Statistical significance of the results were analysis by using GraphPad Prism 6. All values are expressed as mean ± SEM. Statistical significance was determined by one way ANOVA followed by Dunnet post hoc test at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs HYP.

**Results**

**Extractive yield and phytochemical evaluation**

The crude extractive yield of the hydroalcoholic Soxhlet extraction of *S. chirata* and *S. cordata*was observed to be 22.37% and 26.32% respectively. To get an idea of the phytochemicals in the extracts, we performed qualitative phytochemical screening which revealed the presence of alkaloids, flavonoids, terpenoids, glycosides, steroids, tannins and saponins as the major ingredients. Moreover, volatile oil, fixed oil, proteins and acidic compounds were not observed to be present in the plant extracts in significant amounts. We further observed that both extracts were having high amount of flavonoids and phenolic compo unds which might be responsible for its antioxidant potential.

**Behavioral investigation**

**Rota-rod test**

The effect of hypoxia and extract treatments on the muscle coordination was evaluated through Rota-rod test and the results are depicted in Fig. 4.1 (A). Subjecting animals to hypoxia resulted in a nonsignificant impairment in muscle coordination as the time taken to fall from the rotating bar was not significantly different than control animals. Likewise, we did not observed any significant difference in the muscle coordination of control and hypoxic animals who were treated with plant extracts, when compared to CTRL or HYP. These results suggest that short term hypoxia do not impair muscle coordination and animals had normal muscular activity.

**Passive avoidance test**

Effect of *S. chirata* and *S. cordata*extract treatment on the hypoxia mediated memory dysfunction was evaluated through passive avoidance step-through paradigm and the results are depicted in Fig. 4.1 (B). Subjecting animals to hypoxia resulted in a marked memory impairments. Our results demonstrated significantly ($p < 0.001$) lower latency to enter the dark chamber during retention trials, thereby suggesting that animals were not able to remember the foot shock they received in the dark chamber during learning trials. Extract treatment significantly improved memory performance in hypoxic animals. Animals were able to remember the foot shock and were resistant to enter dark chamber. Animals preferred to stay in light chamber and the transfer latencies were significantly ($p < 0.01$) higher than HYP. Further, control animals treated with plant extracts were also able to retain foot shock memory and transfer latencies did not showed any deviations from CTRL.

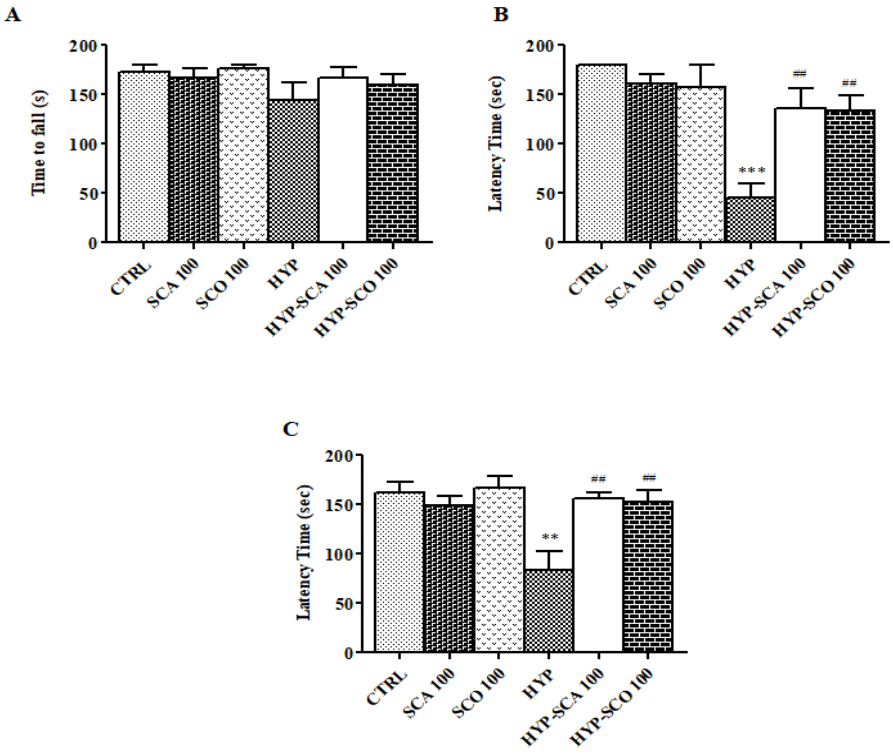


**Fig. 4.1:** Effect of *S. chirata* and *S. cordata*treatment on hypoxia mediated behavioral akterations (A- Rota-rod test; B- Passive avoidance test; C- Active avoidance test). Values are represented mean ± SEM. CTRL: control; SCA 100: control + 100 mg/kg *S. chirata* extract; SCO 100: control + 100 mg/kg *S. cordata* extract; HYP: hypoxia; HYP-SCA 100: HYP + 100 mg/kg *S. chirata* extract; HYP-SCO 100: HYP + 100 mg/kg *S. cordata* extract. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus CTRL group. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus HYP group.

**Active avoidance**

Effect of *S. chirata* and *S. cordata*extract treatment on the hypoxia mediated learning and memory dysfunction was evaluated through active avoidance paradigm and the results are depicted in Fig. 4.1 (C). Hypoxia significantly impaired learning and memory in animals. Hypoxic animals were not able to learn the association between auditory cue and foot sock. These animals demonstrated significantly ($p < 0.01$) higher transfer latency to the safer region of the apparatus, suggesting impaired learning and memory. Extract treatment rescued animals from hypoxia mediated memory dysfunction. These animals efficiently remembered the association between audio cue and foot shock. The transfer latencies of these animals were significantly ($p < 0.01$) lower than the HYP animals. Moreover, control animals treated with plant extracts also demonstrated good learning and memory functions and transfer latencies of these animals did not showed any deviations from CTRL animals.

**Biochemical investigation**

**Mitochondrial ROS Generation**

The results of the mitochondrial ROS generation and the effect *S. chirata* and *S. cordata* treatment are depicted in Fig. 4.2 (A). Hypoxia induced significant oxidative stress in the hippocampus and the levels of hippocampal ROS were observed to be significantly ($p < 0.001$) higher than the CTRL animals. The levels of hippocampal ROS in control animals treated with plant extract did not showed any significant variations from the control animals, suggesting that extract treatment is not contributing directly to enhancing ROS production in hippocampus. Further, treating hypoxic rats with 100 mg/kg dose of *S. chirata* and *S. cordata* extract resulted in a significant ($p < 0.05$) reduction in mitochondrial ROS generation when compared to HYP. Our results suggest that both extracts are having comparable antioxidant potential, which may be attributed to high levels of flavonoids and phenolic compounds present in these plants.

**Lipid Peroxidation**

Lipid peroxidation is a measure of damage to the cellular membrane. The biochemical levels of malondialdehyde (MDA) are estimated as a marker of lipid peroxidation in the biological samples such as brain, especially in the animal experimentation. The results of hypoxia mediated lipid peroxidation and the effects of extract treatments are depicted in Fig. 4.2 (B). Our results suggest that hypoxia induced marked lipid peroxidation in the

hippocampus, as the levels of MDA were observed to be significantly ($p < 0.001$) higher in HYP animals when compared to CTRL. Treating HYP animals with *S. chirata* and *S. cordata* extract reduced lipid peroxidation levels and the levels of MDA were observed to be significantly ($p < 0.001$) lower than HYP. Inhibition of lipid peroxidation was observed to be more pronounced in *S. chirata* treated animals than animals treated with *S. cordata* extract. Moreover, control animals treated with these extracts did not showed any significant difference when compared to CRTL, eliminating any possibility of acute effect of plant extracts.

**Catalase activity**

Catalase is one of the most potent antioxidant enzyme present in our entire body, including brain. It is actively involved in the detoxification of ROS generated during the functioning of electron transport chain. The results of effect of hypoxia and extract treatments in hippocampal catalase levels are depicted in Fig. 4.2 (C). Subjecting animals to hypoxia resulted in the depletion of hippocampal catalase levels. Catalase levels were observed to be significantly ($p < 0.001$) lower in HYP animals, when compared to CTRL animals. Treating hypoxic animals with plant extracts improved hippocampal catalase levels and our results demonstrated a significant ($p < 0.01$) increase in catalase levels, when compared to HYP. Normal animals treated with plant extract did not demonstrated any significant deviation in catalase levels, when compared to CTRL, eliminating any possibility of direct effect of plant extracts. Our results suggest that *S. chirata* and *S. cordata*extracts are having a good antioxidant potential which can be partially attributed to its potential to either upregulate catalase activity or prevent its depletion during oxidative stress.

**Glutathione level**

The effect of hypoxia and extract treatment on hippocampal glutathione levels was evaluated and the results are depicted in Fig. 4.2 (D). Subjecting animals to hypoxia resulted in a significant ($p <0.01$) depletion or lowering of glutathione levels in the hippocampus region of the brain. Our results demonstrated a comparable and marked antioxidant potential of extract treatments. Treating hypoxic animals with *S. chirata* and *S. cordata*extracts significantly ($p < 0.05$) improved hippocampal glutathione levels, when compared to HYP. Normal animals treated with extract did not showed any significant difference in hippocampal glutathione levels, when compared to CTRL, and thereby suggesting observed effects of

treatments during hypoxic conditions are the result of rescuing hippocampus form hypoxia mediated damage.

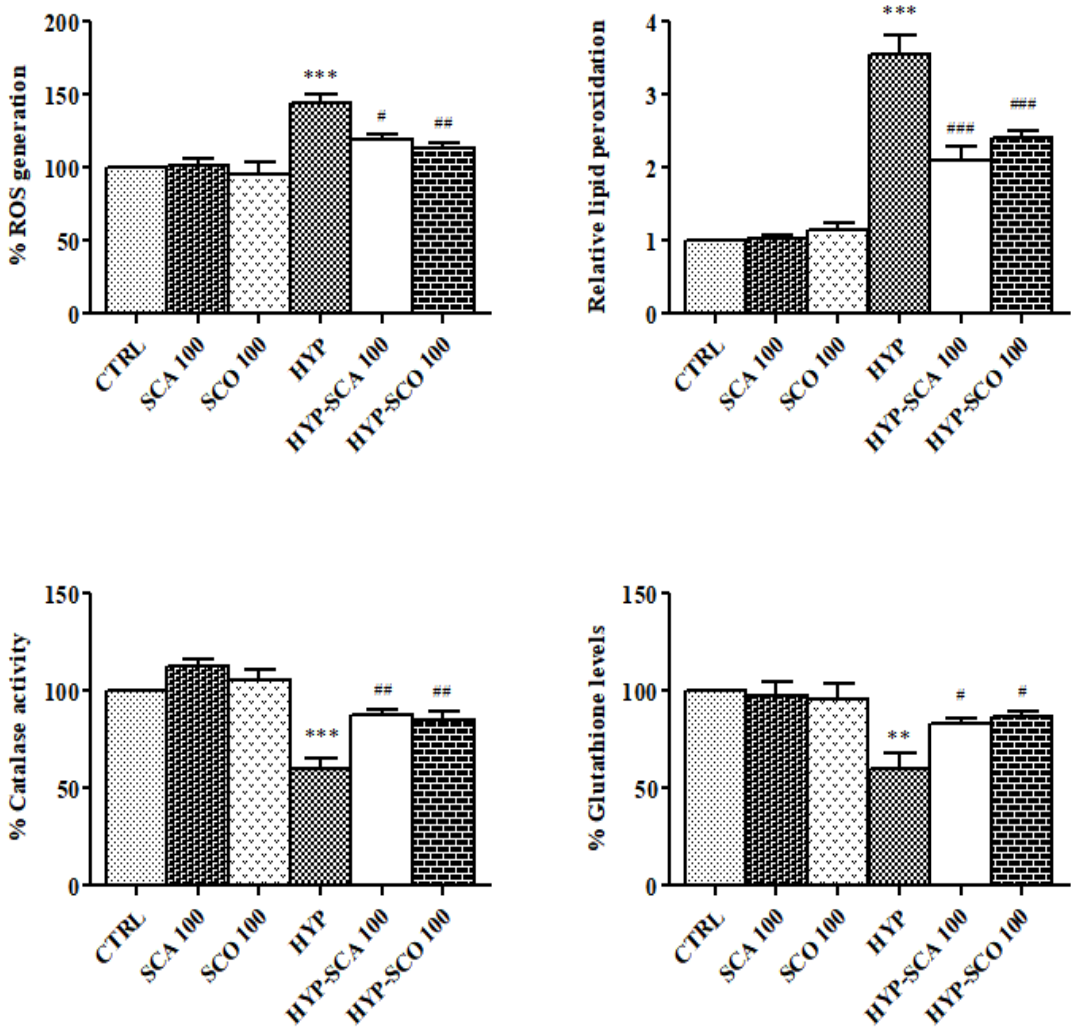


**Fig. 4.2:** Effect of *S. chirata*and *S. cordata*treatment on hypoxia induced oxidative stress (A- % ROS generation; B- lipid peroxidation; C- % catalase activity; D- % glutathione levels). Values are represented mean ± SEM. CTRL: control; SCA 100: control + 100 mg/kg *S. chirata* extract; SCO 100: control + 100 mg/kg *S. cordata* extract; HYP: hypoxia; HYP-SCA 100: HYP + 100 mg/kg *S. chirata* extract; HYP-SCO 100: HYP + 100 mg/kg *S. cordata* extract. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus CTRL group. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus HYP group.

**Histopathological Examination**

Hematoxylin-eosin staining is widely used method to determine the neuronal damage or morphology of neurons. We used this technique to demonstrate the effect of hypoxia on the neuronal morphology and evaluated the neuroprotective effect of *S. chirata* and *S. cordata*extract treatments. Results are depicted in Fig. 4.3. Analysis of the stained sections demonstrated the normal neuronal morphology in samples belonging to CTRL animals. Hypoxia induced marked neuronal damage and neurons appeared to be degenerated or damaged in significant number. Extract treatments rescued neurons from hypoxia mediated damage and neurons of these animals were having normal morphology resembling CTRL animals with lesser signs of damage and degeneration. These results concluded that *S. chirata* and *S. cordata*extract can protect neurons from hypoxia mediated damage, which might have resulted in improved memory functions in these animals.

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**Publications**